Characterization of Disulfide Linkages and Disulfide Bond Scrambling in Recombinant Human Macrophage Colony Stimulating Factor by Fast-Atom Bombardment Mass Spectrometry of Enzymatic Digests

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Fast-atom bombardment mass spectrometry was used to study disulfide bonding patterns in heat-denatured human recombinant macrophage colony stimulating factor (rhM-CSF). The heat-denaturated protein was studied by analysis of the pattern of peptides in the proteolytic digests. Native rhM-CSF is a homodimer with intramolecular disulfide linkages between Cys7–Cys90, Cys48–Cys139, and Cys102–Cys146 and intermolecular linkages between Cys31–Cys31, and the pairs Cys157 and Cys159. Brief heating for 1 min leads to partial disulfide bond scrambling. In addition to the native disulfide bonds between Cys7–Cys90, Cys48–Cys139, and Cys31–Cys31, nonnative disulfide bonds were detected between Cys48–Cys90 and Cys48–Cys102. When heated for 5 min the disulfide bonds of rhM-CSF are completely scrambled and lead to nonnative intramolecular disulfide bonds between Cys48–Cys102 and Cys90–Cys102 and one intermolecular disulfide bond between Cys102–Cys102. *(J Am Soc Mass Spectrom 1995, 6, 638–643)*

Information on the three-dimensional structure of proteins is of fundamental importance to understand their biological activity. Therefore, the analysis of alterations in primary structural features that lead to changes in the higher order structure of proteins are of considerable interest. Our knowledge of these important properties of proteins has improved through analyses by x-ray crystallography and multidimensional NMR techniques [1–4].

A variety of physical techniques, which include circular dichroism and NMR [5, 6], also have been applied to monitor conformational changes in proteins. Recent developments in desorption–ionization sources that allowed for the analyses of large biopolymers also brought about opportunities to probe protein tertiary structures and conformational changes by mass spectrometric methods [7, 8]. An especially powerful technique is electrospray ionization mass spectrometry (ESI-MS), which provides the means to monitor conformational changes in the protein as a whole [9–11]. Proton–deuterium (H/D) exchange reactions in conjunction with mass spectrometric methods have been introduced as chemical probes to analyze protein conformations [12, 13] and higher order structures [14]. In addition, chemical modification of specific amino acid residues and subsequent mass spectrometric analyses have been used to characterize the surface topology of proteins [15, 16].

Disulfide linkages are especially important in determination of protein tertiary structures, and fast-atom bombardment mass spectrometry (FAB-MS) methods have become particularly well established for the rapid analysis of these primary structural features [17, 18]. Recently mass spectrometric methods were used to locate the disulfide linkages (Figure 1a) in recombinant human macrophage colony stimulating factor (rhM-CSF) β [19] and to follow the time-dependent renaturation of the reduced and unfolded monomer [20]. rhM-CSF monomer contains nine cysteines, all of which participate in disulfide bridges when folded correctly, and three of these cysteines form intermolecular bridges that result in a 49-Da homodimer (Figure 1a). In the present study the correctly folded protein was thermally denatured for 1 and 5 min, and then the proteolytic digests were studied by FAB-MS analysis

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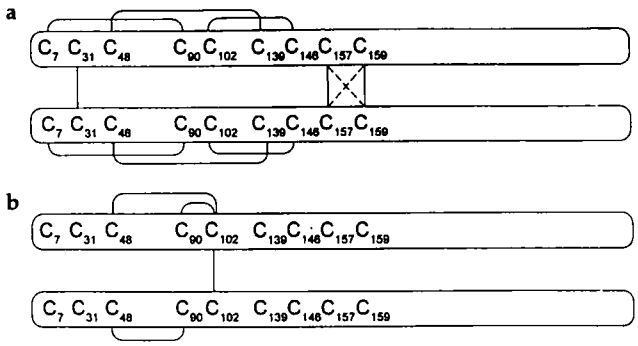

Figure 1. Schematic representations of the rhM-CSF dimers. (**a**) rhM-CSF dimer showing all native intra- and intermolecular disulfide linkages as determined previously [19]. (**b**) Nonnative disulfide bridges presented by the rhM-CSF dimer after denaturation by 5 min of boiling.

to detect changes in the disulfide linkages. We wish to report the results of these investigations.

Materials and Methods

High-Performance Liquid Chromatography Purification and Separations

The high-performance liquid chromatography (HPLC) system consisted of an Altex model 322 gradient liquid chromatograph (Altex, Berkeley, CA) equipped with a Waters 486 tuneable absorbance detector (Waters, Milford, MA) set at 220 nm. Linear gradients were applied by using 0.1% trifluoroacetic acid (TFA) in H_2O as solvent A and 0.08% TFA in CH_3CN as solvent B at a flow rate of 1 mL/min. Protein purification was performed on a Vydac (Hesperia, CA) C4 reversed phase column (30 nm, 10 μm, 4.6 × 250 mm) and peptide separations were carried out on a Vydac C18 reversed phase column (30 nm, 5 μm, 4.6 × 250 mm).

Purification and Buffer Exchange

Purified rhM-CSF was supplied by Chiron Corp. (Emeryville, CA). To exchange buffers the protein was purified by HPLC by using the following gradient. The initial concentration of solvent B was 10%; after 5 min the concentration was increased to 60% B over a period of 60 min and held constant for 10 min. Protein-containing fractions were collected manually, pooled, and lyophilized. The protein was redissolved in 500 μL of 50-mM NH_4HCO_3 to give a protein concentration of 4.5 mg/mL [Biorad (Richmond, CA) protein assay kit that uses bovine serum albumin as standard].

Lys-C Digestion of Native rhM-CSF

Lys-C (Lysyl endopeptidase EC 3.4.21.50, Wako chemicals, Wako Bioproducts, Richmond, VA) solution (1 μL, 1 mg/mL, 333 pmol) dissolved in 50-mM NH_4HCO_3 (pH 8.5) was added to 100 μL of protein solution (0.9 mg/mL) in 50-mM NH_4HCO_3 (pH 8.5; enzyme:substrate = 1:110 w/w). The mixture was incubated for 3 h at 37 °C. Peptides were separated by HPLC and fractions were collected and freeze-dried.

Reduction of Disulfide-Linked Peptides

Aliquots (2.5 μL) of each of the HPLC-purified and redissolved samples that contained disulfide-linked peptides were lyophilized. Samples were redissolved in 30 μL of 50-mM NH_4HCO_3 (pH 8.5) that contained 10-mg/mL dithiothreitol (DTT). After 3 h at 37 °C the samples were freeze-dried.

Reduction, Alkylation and Lys-C Digestion of rhM-CSF

Ammonium bicarbonate (5 μL 50 mM, pH 8.5) solution that contained 20-mg/mL DTT was added to 200 μL of rhM-CSF samples (183 μg, 3.7 nmol) that were dissolved in 50-mM NH_4HCO_3 (pH 8.5). The mixture was incubated for 30 min at 37 °C and then 12-μL 50-mM NH_4HCO_3 (pH 8.5) solution that contained 20-mg/mL iodoacetamide was added. After 1 h, 1% TFA solution was added. Low molecular weight compounds were removed by ultrafiltration via a microconcentrator device (Amicon [Berkeley, MA] Centricon; MW cutoff, 10,000). The buffer was exchanged by ultrafiltration at 4 °C. At this point 300 μL of 50-mM NH_4HCO_3 (pH 8.5) was added and the volume was reduced to 70 μL. This procedure was repeated three times. The final volume was adjusted to 80 μL, which produced a protein concentration of 2 mg/mL. The Lys-C digest was carried out as described above, by using 50 μL of rhM-CSF solution (enzyme:substrate = 1:120 w/w). Peptides were separated by HPLC. Fractions were collected and freeze-dried.

Heat Denaturation and Asp-N and Lys-C Digestion of rhM-CSF

A solution of 5-μL rhM-CSF (28.8 μg, 0.59 nmol), dissolved in 50-mM NH_4HCO_3 (pH 8.5), was heated for 1 min in a water bath at 95 °C. After cooling to room temperature an Asp-N (*Pseudomonas fragi* protease, Boehringer Mannheim, Indianapolis, IN) solution (17 μL, 0.7 μg), dissolved in 10-mM Tris-HCl buffer (pH 7.5), and 2-μl CH_3CN (final conc. approx. 10%) were added (enzyme:substrate = 1:41). The mixture was incubated for 3 h at 37 °C. The reaction was terminated by lyophylization and the sample was subjected to mass spectrometric analysis. A solution of 10-μL rhM-CSF (57 μg, 1.17 nmol), dissolved in 50-mM NH_4HCO_3 (pH 8.5) was heated for 5 min in a water bath at 95 °C. After cooling to room temperature a Lys-C solution (1 μL, 1 mg/mL, 333 pmol), dissolved in 50-mM NH_4HCO_3 (pH 8.5), was added (enzyme:substrate = 1:35 w/w). The mixture was incubated for 3 h at 37 °C. Peptides were separated by HPLC, collected, and lyophylized.

FAB-MS Analysis of HPLC-Separated Peptides and Peptide Mixtures

Purified and lyophilized samples were redissolved in 5-μL 0.1% TFA/H_2O solution. Aliquots (2 μL) of each fraction were mixed on the probe with 2-μL matrix of 3-nitrobenzyl alcohol (3-NBA) or dithiothreitol–dithioerythritol (DTT–DTE; 5:1 v/v). Aliquots (2 μL) of the digests were used without further purification. FAB-MS analysis was carried out on a Kratos MS-50 double-focusing mass spectrometer (Kratas Analytical, Ramsey, NJ) operated at a resolution of 1000. Xenon atoms were produced from an Ion Tech (Teddington, UK) gun operated at 7–8 keV. Ions were accelerated at 8 keV and postaccelerated at 25 keV. The scan rate was 30 s per decade. Molecular weight calculations were carried out using the Kratos DS90 software.

Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry

A previously described [21] custom-built time-of-flight mass spectrometer equipped with a frequency-tripled (355 nm) Nd:YAG laser (Spectra-Physics, Mountainview, CA, GCR-11) was used in the positive ion mode for matrix-assisted laser desorption ionization (MALDI) mass spectrometric analysis of peptides. Accelerating potentials of 24 and 18 kV were applied to the probe and first extraction lens, respectively. The data were generated by 50 consecutive laser pulses. The matrix solution consisted of 4-hydroxy-α-cyanocinnamic acid (HCCA; Aldrich Chemical Co., Milwaukee, WI) in 0.1% TFA/CH_3CN (2:1) at a concentration of 5 g/L. One-microliter of solution of peptides in 0.1% TFA/water (1:1) was mixed with 9 μL of solution of 0.1% TFA/CH_3CN (2:1). This solution (1 μL) was mixed with matrix (1 μL) solution and deposited onto a stainless steel probe. After drying the matrix under a stream of air, the sample was analyzed. Insulin (0.4 pmol; Sigma Chemical Co., St. Louis, MO) was used as an internal calibrant. Mass calibration and data analysis were performed via programs described previously [21].

Results

The protein rhM-CSF, which is expressed in *E. coli* as insoluble and inactive inclusion bodies, has to be renatured by an in vitro refolding process. The final product is obtained after a complex procedure [22] in which the protein undergoes considerable changes in its conformation. The native rhM-CSF and final product of folding are nearly undigestible by proteases. The HPLC trace of a Lys-C digest only showed an early eluting peak, which was identified as peptide L13 (Figure 2a, Table 1), and later eluting peaks that represented minimally digested rhM-CSF. The peaks that eluted prior to L13 gave no signals when analyzed by FAB-MS. Analysis of the unseparated digest exhibited peptide peaks from the C-terminal end (aa164-221, L13, and L14) and from a small portion of the protein that ranged from aa119 to aa137 (L9, L10, and L11). None of the released peptides possessed cysteines (Table 1).

When rhM-CSF was reduced and alkylated with iodoacetamide prior to Lys-C digestion, the HPLC chromatogram revealed additional peaks that represented peptide fragments. Almost no undigested protein remained (Figure 2b). FAB-MS analysis of the unseparated digest showed all expected peptides except L3 and L8 (Table 1). Peptide L6 could not be detected either, but it was identified after HPLC separation in an early eluting fraction. Ionization of these peptides was suppressed in the mixture because of their hydrophilicity. All of the cysteine-containing peptides in the mixture were found to be completely alkylated (Table 1).

The proteolytic digest of heat-denatured rhM-CSF after 5 min boiling yielded an HPLC trace that was more complex than that of the native protein digest (Figure 2c). No undigested protein was detected and some of the same signals observed during analysis of the Lys-C digests of either native or reduced and

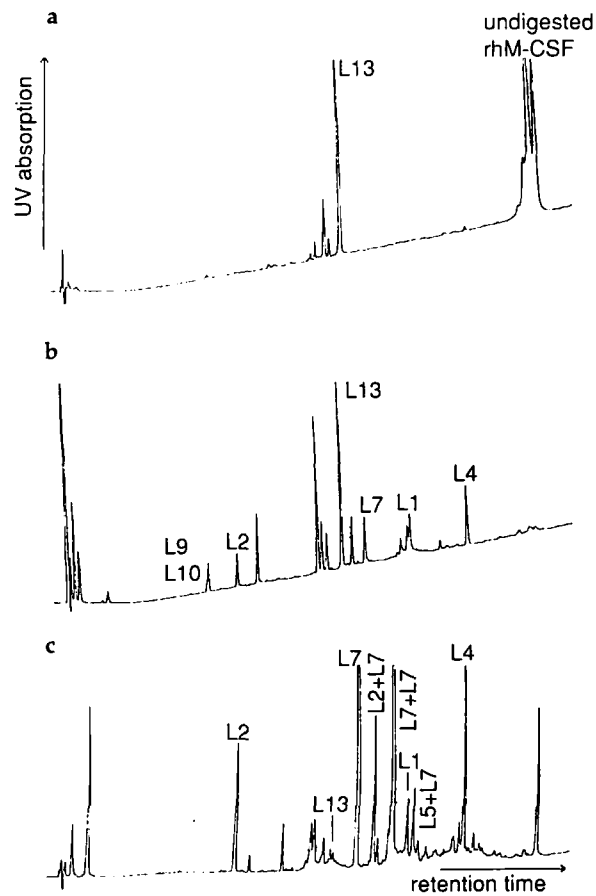

Figure 2. RP-HPLC chromatograms of Lys-C derived peptides from rhM-CSF. (**a**) The Lys-C digest of native rhM-CSF that shows minimally digested protein and L13. (**b**) The Lys-C digest of reduced and thiol-alkylated native rhM-CSF. (**c**) The Lys-C digest of heat-denatured rhM-CSF after 5 min of boiling. Peaks identified by FAB-MS of the fractions are labeled. Peaks that elute just before L13 gave no signals by FAB-MS.

Table 1. FAB-MS analysis of Lys-C derived peptides, thiol-alkylated peptides and disulfide-linked peptides from rhM-CSFβ

Peptide	aa Sequence	Alkyl groups	MH^+ (calc.)[c]	MH^+ (obs.)[a,b] Native	Boiled	Reduced/ alkylated
L1	4-44	0	4746.2	—	4747	—
		1	4803.2	—	—	—
		2	4860.3	—	—	4861
L2	45-51	0	837.4	—	837	—
		1	894.5	—	—	894
L3	52	—	147.1	d	d	d
L4	53-88	—	4202.3	—	4204	4204
L5	89-93	0	585.3	—	585	—
		1	642.3	—	—	642
L6	94-100	—	935.0	d	d	d
L7	101-116	0	1911.0	—	1912	—
		1	1968.1	—	—	1969
L8	117-118	—	246.2	d	d	d
L9	119-125	—	851.4	851	851	851
L10	126-130	—	602.4	602	602	602
L11	131-137	—	909.5	909	909	909
L12	138-163	0	2879.2	—	2880	—
		2	2993.3	—	—	—
		3	3050.4	—	—	—
		4	3107.4	—	—	3108
L13	164-218	—	5502.7	5505	5505	5505
L14	219-221	—	400.2	400	400	400
L2 + L7	(45-51) + (101-116)	—	2745.4	—	2747	—
L5 + L7	(89-93) + (101-116)	—	2493.2	—	2494	—
L7 + L7	(101-116) + (101-116)	—	3819.0	—	3820	—

[a] Rounded-off values.
[b] The observed masses above m/z 1400 are higher than the calculated monoisotropic values because of a shift in the center of mass in the isotopic cluster.
[c] Monoisotopic masses.
[d] Not observed due to suppression of the ion.

alkylated sample were observed here again. In addition this denatured protein gave signals for disulfide-linked peptides (Figure 2c, Table 1). Peaks were assigned by comparison of retention times and by FAB-MS analyses [19]. Thus, the HPLC fraction that contained the disulfide-linked peptide L5 + L7 found at m/z 2494 (and K^+ adducts) was partially reduced upon sample workup in DTT and yielded peptide L7, which appears at m/z 1912 in the mass spectrum (Figure 3a). After complete reduction with DTT, the peak at m/z 2494 disappeared entirely and the peaks for the separated peptides L7 and L5 remained (Figure 3b). This shows that Cys90 was connected to Cys102 (Figure 1b). In native rhM-CSF, Cys90 is connected to Cys7 and Cys102 to Cys 146 (Figure 1a).

After boiling the protein for 5 min, three disulfide bridges were detected by FAB-MS analysis in the mass range between m/z 2300 and 5000 (Figure 4a). Cys102 was involved in all of the disulfide linkages. In 3-NBA, a nonreducing matrix, peaks were recorded at m/z 2747, 2494, and 3820 for these disulfide-linked peptides. There also were peaks at m/z 4747 for L1, m/z 4204 for L4, and m/z 2880 for L12. All peaks appeared as doublets due to the formation of K^+ adducts. L1 and L12 contain two and four cysteines each, but these peptide peaks can only appear at m/z 4747 and 2880, respectively, when no disulfide bonds are present. After complete reduction peaks at m/z 2747, 2494, and 3820 disappeared, which confirmed that these signals in the spectra of the unreduced sample were caused by disulfide-linked peptides. Only signals for peptides L1, L4, L12, and an unidentified peak at m/z 3586 remained in this mass range (Figure 4b). Experiments by MALDI time-of-flight mass spectrometry showed no additional peaks in the mass range to m/z 7000.

Further studies revealed that brief heating for only 1 min at 95 °C leads to partial scrambling of the disulfide bonds in rhM-CSF. The samples were digested with Asp-N, which cleaves at the *N*-terminal side of aspartyl residues (Figure 5) and sometimes nonspecifically at glutamyl, theronyl, and seryl residues [23]. FAB-MS analysis of an Asp-N digest mixture showed the presence of the native disulfide bonds between Cys7–Cys90, Cys31–Cys31, and

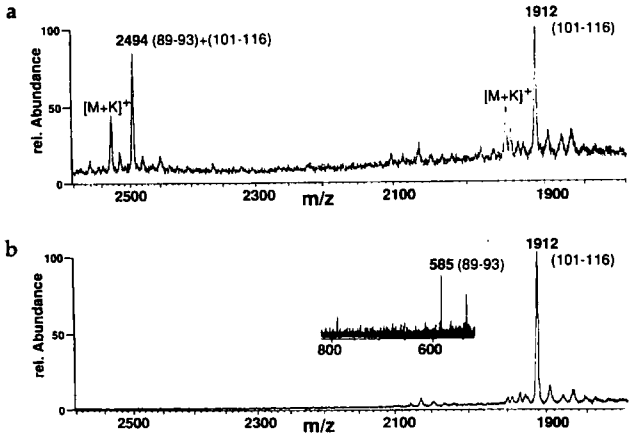

Figure 3. FAB-MS of HPLC fraction L5 + L7 after denaturation of rhM-CSF by boiling (see Figure 2c). (**a**) Partially reduced sample. (**b**) Sample after complete reduction. In both cases DTT-DTE was used as the matrix. The numbers in parentheses correspond to amino acids in the peptides.

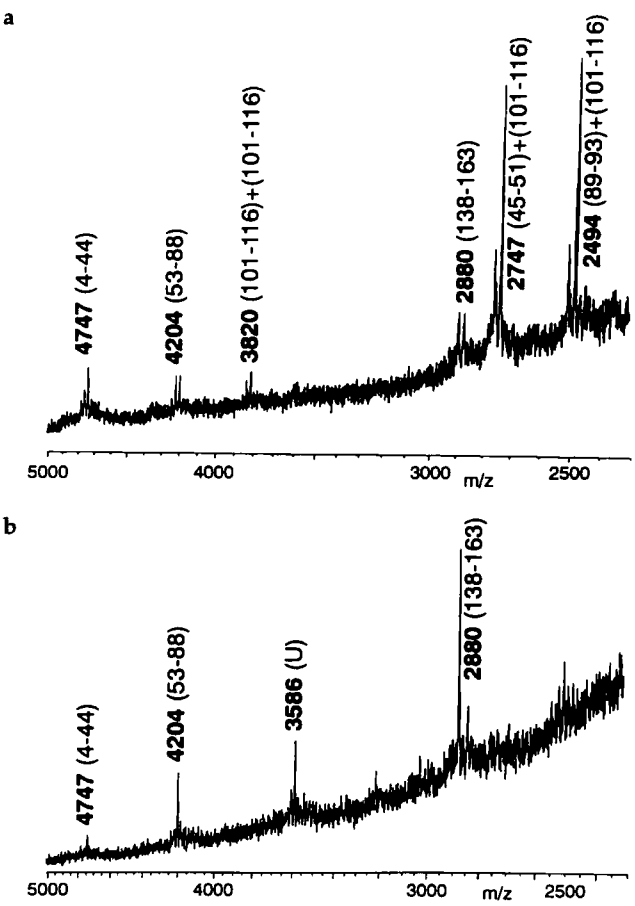

Figure 4. FAB-MS in the mass range 2300-5000 of a Lys-C derived peptide mixture of rhM-CSF after denaturation by 5 min of boiling. (**a**) Spectrum of nonreduced peptides in 3-NBA. (**b**) Spectrum after complete reduction. The spectrum was recorded in DTT-DTE matrix. U = unidentified peak. The numbers in parentheses correspond to amino acid residues in the peptides.

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        10          20          30          40
SEYCSHM     IGSGHLQSLQ  RLIDSQMETS  CQITFEFVDQ
L1

        50          60          70          80
EQLKDPVCYL  KKAFLLVQDI  MEDTMRFRDN  TPNAIAIVQL
L1          L2

        90          100         110         120
QELSLRLKSC  FTKDYEEHDK  ACVRTFYETP  LQLLEKVKNV
L4          L5  L6                  L7  L8

        130         140         150         160
FNETKNLLDK  DWNIFSKNCN  NSFAECSSQD  VVTKPDCNCL
L9  L10     L11         ▷ L12

        170         180         190         200
YPKAIPSSDP  ASVSPHQPLA  PSMAPVAGLT  WEDSEGTEGS
L12

        210         220
SLLPGEQPLH  TVDPGSAKQR  P
            L13     L14
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Figure 5. rhM-CSF amino acid sequence. The numbering of residues is that of the native species. Cleavages for Lys-C and selected cleavages for Asp-N are shown and the resulting peptides are assigned [19].

Cys48-Cys139 by mass peaks at m/z 3682, 3527, and 3294, respectively (Figure 6a). In addition the presence of nonnative disulfide bonds in this sample is shown by peaks at m/z 3509 and 2737, which are formed by linkages between Cys48-Cys90 and Cys48-Cys102, respectively. The nonreducing matrix 3-NBA was used in this experiment and resulted in a poor signal-to-noise ratio for the signals of interest. The unambiguous identification of the disulfide-linked peptides, however, was confirmed by MALDI mass spectrometry experiments that used this sample. After complete reduction with DTT as a matrix (Figure 6b), all peaks disappeared, which confirmed that the signals resulted from disulfide-linked peptides.

These results indicate that rhM-CSF alters its conformation when heated and then exhibits a different behavior toward proteolysis. The change in conformation leads to the formation of nonnative disulfide bonds via disulfide bond scrambling, which opens up the protein sufficiently to allow it to be digested without prior reduction. The disulfide bonds exhibited by rhM-CSF in native and in heat-denatured conformations are totally different (Figure 1).

Discussion

Altering the tertiary structure of a protein by heat denaturation is a process that occurs without changes in the sequence. Thus, proteolytic enzymatic methods are able to differentiate between alternative disulfide-linked structures in which the sequence is the same because these different structures can lead to different digestion products, which coincidentally also mirror the changed conformation. Disulfide bond reduction and thiol-alkylation of the free cysteines is frequently used prior to primary structural analyses, because in

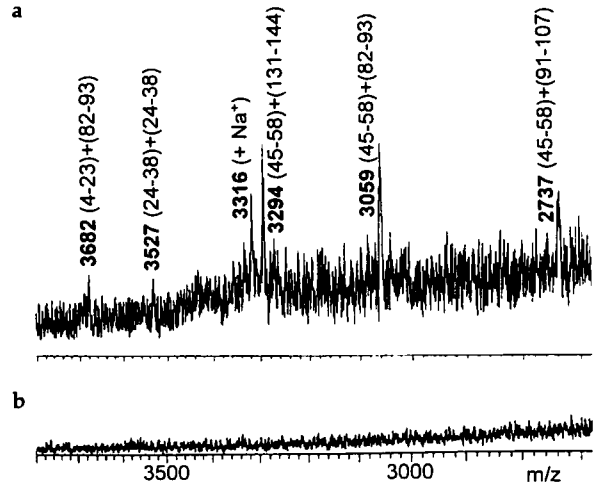

Figure 6. FAB-MS in the mass range 2300–3800 of an Asp-N derived peptide mixture of rhM-CSF after denaturation by 1 min of boiling. (**a**) Spectrum of nonreduced peptides in 3-NBA. (**b**) Spectrum after complete reduction. The spectrum was recorded in DTT–DTE matrix.

the thiol-alkylated form of the protein, renaturation is not possible and all proteolytic cleavage sites are accessible. This allows for complete digestion and determination of primary structural features.

Native rhM-CSF (Figure 5) is very resistant to proteolytic cleavage and only fragments from its C-terminus (aa163–221) and from a small section of the interior region (aa119–137) are released upon Lys-C digestion [19]. This behavior is also characteristic of rhM-CSF in its refolded form, but only many hours after all disulfide bonds have formed. Aging appears to be a requirement to obtain a proteolysis-resistant structure [20]. In native structures the resistance to proteolysis initially prevented the assignment of the disulfide bridges by conventional methods. Most of the proteolytic cleavage sites were inaccessible to proteases in this tightly folded tertiary protein structure.

In the case of rhM-CSF, heat denaturation leads to a major change in the disulfide linkages and a totally different behavior to proteolysis. Disulfide bond scrambling occurs and locks the protein into new conformations. After only 5 min boiling complete reduction of some of the disulfide bonds, for example, L12 that contains four cysteines, was observed (Figure 3). The disulfide bridges observed after boiling were nonnative and involved intra- and intermolecular disulfide bonds to Cys102 (Figure 1b).

Acknowledgments

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